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(54) Title: NUCLEIC ACID SEQUENCES SHOWING ENHANCED EXPRESSION IN BENIGN NEUROBLASTOMA COMPARED WITH ACRITICAL HUMAN NEUROBLASTOMA

(54) 発明の名称: 予後良好及び不良なヒト神経芽細胞腫との比較において、予後良好なヒト神経芽細胞腫で発現が 増強していることを特徴とする核酸配列

(57) Abstract: Nucleic acids originating in a gene which is expressed in human neuroblastoma, characterized by showing enhanced expression in benign human neuroblastoma compared with in acritical human neuroblastoma and having a sequence selected from among the group consisting of the nucleic acid sequences represented by SEQ ID NOS:1 to 104 in Sequence Listing; nucleic acids complementary with the above nucleic acids; fragments of these nucleic acids; use thereof as a probe or a primer; and diagnosis of the prognosis of human neuroblastoma with the use of any of the same.

(57) 要約:

ヒト神経芽細胞腫において発現する遺伝子に由来する核酸であって、特に予後良好及び不良なヒト神経芽細胞腫との比較において、予後良好なヒト神経芽細胞腫で発現が増強していることを特徴とし、かつ配列表の配列番号1ないし104に記載の核酸配列からなる群より選ばれる1つの配列からなることを特徴とする核酸またはそれに相補的な核酸、およびそれら核酸の断片、並びにそれらのプローブ或いはプライマーとしての使用、さらにそれらのいずれかを用いるヒト神経芽細胞腫の予後の診断が開示される。

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(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI 特許 (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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# 添付公開書類:

-- 国際調査報告書

# 明細書

予後良好及び不良なヒト神経芽細胞腫との比較において、予後良好なヒト神経芽細胞腫で発現が増強していることを特徴とする核酸配列

# 5 技術分野

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本発明は、ヒト神経芽細胞腫において発現する遺伝子に由来する核酸類に関する。さらに詳しくは、本発明は、予後良好なヒト神経芽細胞腫と、予後不良なヒト神経芽細胞腫との比較において、予後良好なヒト神経芽細胞腫で発現が増強されている遺伝子に由来する核酸およびその断片、並びにヒト神経芽細胞腫の予後の診断へのその用途に関する。

# 背景技術

個々の腫瘍にはそれぞれの個性があり、発癌の基本的な原理は同じであっても、 その生物学的特性は必ずしも同じではない。近年、癌の分子生物学や分子遺伝学 が急速に進歩し、発癌やいわゆる腫瘍細胞のバイオロジーが遺伝子レベルで説明 できるようになってきた。

#### (神経芽細胞腫)

神経芽細胞腫は、末梢交感神経系細胞に由来する交感神経節細胞と副腎髄質細胞に発生する小児癌である。この交感神経系細胞は、発生初期の神経堤細胞が腹側へ遊走し、いわいる交感神経節が形成される場所で分化成熟したものである。

20 その一部の細胞は、さらに副腎部へ遊走し、先に形成されつつある副腎皮質を貫通して髄質部に達し、そこで髄質を形成する。神経堤細胞は、ほかの末梢神経細胞の起源ともなっており、後根神経節(知覚神経)、皮膚の色素細胞、甲状腺C細胞、肺細胞の一部、腸管神経節細胞などへ分化する。

# (神経芽細胞腫の予後)

25 神経芽細胞腫は多彩な臨床像を示すことが特徴である(中川原:神経芽腫の発生とその分子機構 小児内科 30,143, 1998)。例えば、1歳未満で

発症する神経芽細胞腫は、非常に予後が良く、大部分が分化や細胞死を起こして自然退縮する。現在、広く実施されている生後6か月時の尿のマススクリーニングで陽性となる神経芽細胞腫の多くは、この自然退縮を起こしやすいものに属する。一方、1歳以上で発症する神経芽細胞腫は、悪性度が高く、多くの場合、患児を死に至らしめる。1歳以上の悪性度の高い神経芽細胞腫は、体細胞突然変異(Somatic mutation)が起こり、モノクローナルであるのに対し、自然退縮する神経芽細胞腫では、生殖細胞突然変異(germline mutation)のみの遺伝子変異でとどまっているとの仮説もある。Knudson AG等:Regression of neuroblastoma IV-S:A genetic hypothesis. N Engl J Med 302, 1254 (1980)を参照。

(神経芽細胞腫の予後診断を可能にする腫瘍マーカー)

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最近の分子生物学的研究の進展により、神経成長因子 (nerve grow th factor:NGF)の高親和性レセプターであるTrkAの発現が分 化と細胞死の制御に深くかかわっていることが明らかとなってきた。Nakag awara A., The NGF story and neuroblas Med Pediatr Oncol, 31, 1 1 3 (19 98)を参照。Trkは膜貫通型レセプターでもあり、Trk-A、B、Cの3 つが主なものである。これらTrkファミリー・レセプターは、中枢神経および末 梢神経系において、特異的な神経細胞の分化と生存維持に重要な役割を果たして いる。中川原等:神経芽細胞腫におけるニューロトロフィン受容体の発現と予後 小児外科 29:425-432,1997を参照。ところで、腫瘍細胞の生存 や分化は、TrkチロシンキナーゼやRetチロシンキナーゼからのシグナルで 制御されている。なかでも、TrkAレセプターの役割は最も重要で、予後良好 な神経芽細胞腫ではTrkAの発現が著しく高く、これからのシグナルが腫瘍細 胞の生存・分化、または細胞死 (アポトーシス) を強く制御している。一方、予

後不良な神経芽細胞腫では、TrkAの発現が著しく抑えられており、これに代わってTrkB或いはRetbのシグナルが生存の促進という形で腫瘍の進展を助長している。

また、神経の癌遺伝子であるN-mycの増幅が神経芽細胞腫の予後に関連していることも明らかになってきた。中川原:脳・神経腫瘍の多段階発癌,Molecular Medicine,364,366 (1999)を参照。この遺伝子は神経芽細胞腫で初めてクローニングされたが、正常細胞や予後良好な神経芽細胞腫では通常1倍体当たり1つしか存在しないのに対し、予後不良の神経芽細胞腫においては数十倍に増幅されているのが見つかった。このようにN-mycの増幅は、腫瘍の進行度に深く関係している。

しかしながら、現在までに、神経芽細胞腫に発現されている癌遺伝子は、Nーmyc以外知られておらず、その予後の良不良に関する遺伝子情報に関しても、N-mycとTrKA以外はほとんど知られていなかった。

#### 発明の開示

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本発明は、かかる事情に鑑みてなされたものであり、神経芽細胞腫において発現する遺伝子の情報を明らかにし、さらに予後の良不良に関係する前記遺伝子の情報をも明らかにし、それらの遺伝子情報に基づいて、神経芽細胞腫の予後の良不良に関する診断を可能とすることを目的とする。

本発明者は上記目的に従い、鋭意研究を重ねた結果、ヒト神経芽細胞腫の予後を検定し、予後良好および予後不良の臨床組織の各々から c D N A ライブラリーを作製することに成功した。これらの2種類の c D N A ライブラリーから各々約2400クローンをクローニングし、神経芽細胞腫の予後の良悪によって分類した。

また、本発明者は、前記クローニングされた遺伝子の部分または全長をシーク エンシングし、さらにホモロジー検索を行って、適当な遺伝子を選出した。

さらに、本発明者は、上記のように分類した遺伝子群を前記選出した遺伝子に

着目して比較すると、かなりの数の遺伝子において、神経芽細胞腫の予後良好な 臨床組織でのみ発現が増強していることを見いだした。

かかる知見に基づき、本発明者は、ヒト神経芽細胞腫の予後良好な臨床組織でのみ発現が増強している遺伝子を検出およびクローニングするための遺伝子情報 (核酸配列情報等)を提供することを可能とした。さらに、前記核酸配列情報に基づき、予後同定の方法およびそのために使用可能な腫瘍マーカーを設計することを可能とし、本発明を完成した。

すなわち本発明は、下記1~8に記載の核酸または核酸断片を提供する。さらに、本発明は、下記9~11に記載の該核酸または核酸断片の用途を提供する。

- 1. ヒト神経芽細胞腫において発現する遺伝子に由来する核酸であって、配列表の配列番号1ないし104に記載の核酸配列からなる群より選ばれる1つの配列からなることを特徴とする核酸、またはそれに相補的な核酸。
  - 2. 前記核酸がDNAであることを特徴とする上記1に記載の核酸。
- 3. 予後良好なヒト神経芽細胞腫と、予後不良なヒト神経芽細胞腫との比較において、予後良好なヒト神経芽細胞腫で発現が増強されている遺伝子に改来し、配列表の配列番号1ないし104に記載の核酸配列からなる群より選ばれる1つの配列からなることを特徴とする核酸、またはそれに相補的な核酸。
  - 4. 前記核酸がDNAであることを特徴とする上記3に記載の核酸。
  - 5. 上記1~4のいずれか1つに記載の核酸の断片。

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- 20 6. 上記1~4のいずれか1つに記載の核酸とストリンジェントな条件下でハイブリダイズすることを特徴とする、単離された核酸。
  - 7. 前記核酸がDNAであることを特徴とする上記6に記載の単離された核酸。
  - 8. 上記7に記載の核酸からなることを特徴とするPCRプライマー。
  - 9. 上記3に記載の核酸をヒト神経芽細胞腫の臨床組織から検出することを特徴とする、ヒト神経芽細胞腫の予後の診断方法。
  - 10.上記8に記載のPCRプライマーの一組を含むことを特徴とするヒト神経

芽細胞腫の予後の診断用キット。

従って、上記の好ましい核酸は、予後良好なヒト神経芽細胞腫と、予後不良なヒト神経芽細胞腫との比較において、予後良好なヒト神経芽細胞腫でのみ発現が増強されている遺伝子に由来するものであり、該核酸の配列に関する情報はヒト神経芽細胞腫の予後の診断を可能にすることを特徴とする。

# 図面の簡単な説明

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図1は、予後良好・不良ヒト神経芽細胞腫での遺伝子発現量を半定量的PCRで調べた結果、予後良好なヒト神経芽細胞腫での発現の増強が認められた遺伝子の一例(核酸配列nbla-00106からの結果)を示す電気泳動写真に対応する図である。図中、レーン1~16は、予後良好なヒト神経芽細胞腫の臨床組織の試料である。一方、レーン17~32は、予後不良なヒト神経芽細胞腫の臨床組織の試料である。

図2は、予後良好・不良ヒト神経芽細胞腫での遺伝子発現量を半定量的PCRで調べた結果、予後良好なヒト神経芽細胞腫での発現の増強が認められた遺伝子の別の例(核酸配列nbla-00219からの結果)を示す電気泳動写真に対応する図である。図中、レーン1~16は、予後良好なヒト神経芽細胞腫の臨床組織の試料である。一方、レーン17~32は、予後不良なヒト神経芽細胞腫の臨床組織の試料である。

図3は、予後良好・不良ヒト神経芽細胞腫での遺伝子発現量を半定量的PCRで調べた結果、予後良好なヒト神経芽細胞腫での発現の増強が認められた遺伝子のさらに別の例(核酸配列nbla-03145からの結果)を示す電気泳動写真に対応する図である。図中、レーン1~16は、予後良好なヒト神経芽細胞腫の臨床組織の試料である。一方、レーン17~32は、予後不良なヒト神経芽細胞腫の臨床組織の試料である。

図4は、細胞周期特異的な遺伝子発現を半定量的PCRで調べた結果、該発現 が認められた遺伝子の一例(核酸配列nbla-00100からの結果)を示す

# 発明を実施するための最良な形態

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以下、本発明に係るヒト神経芽細胞腫に発現する遺伝子(以下、「本発明に係る遺伝子」という)に由来する核酸およびそれに関連する核酸断片について(以下、「本発明の核酸」および「本発明の核酸断片」というが、特に核酸とその断片を

区別して、記載する必要のないとき、それらを集合的に「本発明の核酸」ともいう)、本発明の好適な実施の形態を参照して、詳細に説明する。

本発明の核酸は、上述のごとく本発明に係る遺伝子に由来するものであり、該遺伝子を構成するか或いは該遺伝子からインビボまたはインビトロの過程によって得られる。そこで、本明細書で使用する「核酸」という用語は、例えばDNAまたはRNA、或いはそれから誘導された活性なDNAまたはRNAであるポリヌクレオチドを指し、好ましくは、DNAまたはRNAを意味する。特に好ましい核酸は、本明細書中に開示されるヒトcDNA配列と同一か、または相補的な配列を有する。

また、本明細書で使用する「ストリンジェントな条件下でハイブリダイズする」 という用語は、2つの核酸(または断片)が、サムブルックら(Sambroo k, J.)の「大腸菌におけるクローン遺伝子の発現(Expression

of cloned genes in E. coli)」、Molecular Cloning:A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press, New York, USA, 9. 47-9. 62および11. 45-11. 61に

記載されたハイブリダイゼーション条件下で、相互にハイブリダイズすることを 意味する。

より具体的には、前記「ストリンジェントな条件」とは、約45℃において6.0×SSCでハイブリダイゼーションを行った後に、50℃において2.0×SSCで洗浄することを指す。ストリンジェンシーの選択のため、洗浄工程における塩濃度を、例えば低ストリンジェンシーとしての約2.0×SSC、50℃から、高ストリンジェンシーとしての約0.2×SSC、50℃まで選択すること、ができる。さらに、洗浄工程の温度を低ストリンジェンシー条件の室温、約22℃から、高ストリンジェンシー条件の約65℃まで増大させることもできる。

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また、本明細書で使用する「単離された核酸」という用語は、組換えDNA技術により作成された場合は細胞物質、培養培地を実質的に含有せず、化学合成された場合には前駆体化学物質またはその他の化学物質を実質的に含まない、核酸またはポリヌクレオチドを指す。

また、本明細書で使用する「予後良好」とは、ヒト神経芽細胞腫のうち、腫瘍が限局して存在するか、または退縮や良性の交感神経節細胞腫になった状態を指し、これはN-mycその他の腫瘍マーカー(TrkA、染色体異常等)から判断して、悪性度が低いと医師によって判断される。本発明の好適な実施の形態では、病期1または2、発症年齢が1歳未満、手術後5年以上再発なく生存し、臨床組織中にN-mycの増幅が認められない症例を予後良好としたが、このような特定の例には限定されない。また、本明細書で使用する「予後不良」とは、ヒト神経芽細胞腫のうち、腫瘍の進行が認められる状態を指し、これはN-mycその他の腫瘍マーカーから判断して、悪性度が高いと医師によって判断される。本発明の好適な実施の形態では、病期4、発症年齢が1歳以上、手術後3年以内に死亡、臨床組織中にN-mycの増幅が認められた症例を予後不良としたが、このような特定の例には限定されない。

神経芽細胞腫は、ヒトでは2種類しか知られていない神経細胞そのものの腫瘍

の1つであり、そこで発現している遺伝子を解析することは、神経細胞のバイオロジーを理解する上で非常に有用な知見をもたらすものと考えられる。すなわち、脳や末梢神経から、部位特異的な均質な組織を得ることは極めて困難で、事実上不可能である。一方、神経芽細胞腫は、末梢交感神経細胞に由来するほぼ均一な神経細胞集団(腫瘍化してはいるが)から成り、均質に発現している神経関連遺伝子が得られる可能性が高い。また、神経芽細胞腫は癌であるため、神経発生の未熟な段階で発現している重要な遺伝子が多いことも特徴として挙げられる。

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さらに、神経芽細胞腫は、予後の良好なものと予後の不良なものとが臨床的、生物学的にはっきり区別される。予後良好な神経芽細胞腫の癌細胞は、増殖速度が極めて遅く、ある時点から自然退縮を始めることが特徴である。これまでの知見から、この自然退縮では、神経細胞の分化およびアポトーシス(神経細胞死)が起こっており、正常神経細胞の成熟段階で起こる分化とプログラム細胞死と非常によく似た現象であることが分かってきた。従って、この腫瘍で発現している遺伝子を解析することによって、神経の分化やアポトーシスに関連した重要な遺伝子情報を入手できる可能性が極めて高い。

予後不良な神経芽細胞腫は、明らかに悪性増殖を続ける癌細胞からなる腫瘍である。従って、神経細胞の増殖に関連した重要な遺伝子や、未分化な神経細胞で発現している遺伝子が多数存在する可能性が高い。すなわち、予後良好な神経芽細胞腫で発現している遺伝子のプロファイルとは全く異なる遺伝子情報を入手できる可能性が極めて高い。

一般的に神経細胞は、他の臓器由来の細胞と比較して、発現している遺伝子の 種類が多いと言われている。神経芽細胞腫の細胞株(セルライン)は、予後不良 の臨床組織由来であり、腫瘍化に伴い遺伝子発現のプロファイルが正常神経細胞 と大きく変化しているものと考えられる。

25 また、神経芽細胞腫は小児由来の腫瘍であることが1つの特徴であり、後天的 な囚子の影響が非常に少ない可能性が高く、癌発生のメカニズムの解析とともに

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発生学的な情報を入手できる可能性が高いことも予想される。さらに驚くべきこ とには、本発明の核酸の中に、ある特定の細胞周期にのみ発現が増強する遺伝子 に由来する核酸が含まれており、このことからも瘍発生のメカニズムの解析およ び発生、分化に関する非常に有用な遺伝子情報を入手できる可能性が高いことが 予想される。

上記のような特徴を有し、有用な遺伝子情報を入手できる遺伝子に由来する核 酸である本発明の核酸は、ヒト神経芽細胞腫の臨床組織より得られ、配列表の配 列番号1ないし104に記載の核酸配列のうちのいずれか1つ、またはその核酸 配列の一部を有する。

10 さらに、ヒト神経芽細胞腫の予後良好なものと、不良なものとの臨床組織にお ける本発明に係る遺伝子の発現量を比較した結果、配列番号1ないし104に記 載の各核酸配列に対応する遺伝子の全てにおいて非常に顕著な差が認められた。 すなわち、これらの遺伝子は、予後良好なヒト神経芽細胞腫で発現が増強されて いた。従って、配列番号1ないし104に記載の核酸配列は、上記の有用な遺伝 子情報以外に、それらの核酸配列のいずれかを有する核酸(DNAまたはRNA) を検出することによって神経芽細胞腫の良不良を診断する腫瘍マーカーの情報と しても利用可能である。

すなわち、本発明は、ヒト神経芽細胞腫およびそれに関連する様々な遺伝子情 報を以下の手段によりうることを可能とする。

20 (1) ハイブリダイゼーションに用いるプローブ

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本発明の1つの実施の形態に従えば、本発明の核酸またはその断片をハイブリ ダイゼーションのプローブとして使用することによって、ヒト神経芽細胞腫で発 現している遺伝子を検出することが可能である。さらに、本発明の核酸またはそ の断片をハイブリダイゼーションのプローブとして使用し、様々な腫瘍、正常組 織における遺伝子発現を調べることによって、該遺伝子発現の分布を同定するこ とも可能である。

本発明の核酸またはその断片をハイブリダイゼーションのプローブとして使用する場合、ハイブリダイゼーション法自身については、特に限定はない。好適な方法として、例えば、ノザンハイブリダイゼーション、サザンハイブリダイゼーション、ドットハイブリダイゼーション、Fluorescence in situ hybridization (FISH)、in situ hybridization (ISH)、DNAチップ法、マイクロアレイ法、等が挙げられる。

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前記ハイブリダイゼーションの1つの応用例として、本発明の核酸またはその断片をノザンハイブリダイゼーションのプローブとして用い、検定したい試料中においてmRNAの長さを測定することや、遺伝子発現を定量的に検出することが可能である。

また別の応用例として、本発明の核酸またはその断片をサザンハイブリダイゼーションのプローブとして用い、検定したい試料のゲノムDNA中、該DNA配列の有無を検出することが可能である。

15 さらに別の応用例として、本発明の核酸またはその断片をFISH法のプローブとして用い、遺伝子の染色体上の位置を同定することも可能である。

さらに別の応用例として、本発明の核酸またはその断片をISH法のプローブ として用い、遺伝子の発現の組織分布を同定することも可能である。

本発明の核酸またはその断片をハイブリダイゼーション用プローブとして使用する場合、少なくとも40個の核酸残基長が必要であり、本発明の核酸またはその断片のうち、40個以上の連続した残基があるものが好ましく用いられる。さらに好ましくは、60個以上の残基を有するものが用いられる。

当業者にとって、上記各種のハイブリダイゼーションにおける核酸プローブ技法は周知であり、例えば、個々の長さの本発明に係る核酸プローブと、目的とするポリヌクレオチドとの適当なハイブリダイズ条件は容易に決定することができる。種々の長さを含むプローブに対し至適なハイブリダイズ条件を得るためのか

かる操作は、当業者では周知であり、例えばサンブルックら、Molecular Cloning: A Laboratory Manual (前掲)を参照して、行えばよい。

好ましくは、本発明に係るプローブは、容易に検出されるように標識される。 検出可能な標識は、目視によって、または機器を用いるかのいずれかによって検出され得るいかなる種類、元素または化合物であってもよい。通常使用される検出可能な標識としては、放射性同位元素、アビジンまたはビオチン、蛍光物質(FITCまたはローダミン等)が挙げられる。前記放射性同位元素は、32P、14C、125I、3H、35S等である。また、ビオチン標識ヌクレオチドは、ニックトランスレーション、化学的または酵素的手段によって、核酸に組み込むことができる。ビオチン標識されたプローブは、アビジン/ストレプトアビジン、蛍光標識、酵素、金コロイド複合体等などの標識手段を使用したハイブリダイゼーションの後検出される。また、本発明に係るプローブは、タンパク質と結合させることによって標識されてもよい。その目的で、例えば放射性または蛍光ヒストンー本鎖結合タンパク質が使用される。

#### (2) PCRに用いるプライマー

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目的遺伝子(例えば、本発明に係る遺伝子)を検出するには上記のハイブリダイゼーション法の他に、本発明の核酸またはその断片に含まれる任意の核酸(DNA)配列をプライマーとして、Polymerase Chain Reaction (PCR) 法を用いることにより可能である。例えば、検定したい臨床組織試料からmRNAを抽出し、RT-PCR法により遺伝子発現を半定量的に測定することが可能である。このような方法は、当業者にとって周知の方法に従って行われるが、例えば、サンブルックら、Molecular Cloning: A Laboratory Manual (前掲)、および遺伝子病入門(高久史麿著:南江堂)が参照される。

本発明の核酸またはその断片をPCR用プライマーとして使用する場合、10

ないし60個の核酸残基長が必要であり、本発明の核酸またはその断片のうち、10ないし60個の連続した残基があるものが好ましく用いられる。さらに好ましくは、15ないし30個の残基を有するものが用いられる。また一般的には、プライマー配列中のGC含量が40ないし60%のものが好ましい。さらに、増幅に用いる2つのプライマー間のTm値に差がないことが望まれる。また、プライマーの3、末端でアニールせず、プライマー内で2次構造をとらないことも望ましい。

# (3)遺伝子のスクリーニング

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本発明の核酸またはその断片を使用することによって、様々な組織や細胞で発現している目的遺伝子の発現分布を検出することが可能である。これは例えば、本発明の核酸またはその断片を上記のようにハイブリダイゼーションのプローブ、またはPCRのプライマーとして使用することによって、可能となる。

また、DNAチップ、マイクロアレイ等を用いても目的遺伝子の発現分布を検出することが可能である。すなわち、本発明の核酸またはその断片を直接、前記チップ、アレイ上に張り付けことが出来る。そのため高精度分注機でかかる核酸等(DNA)を基板にスポットする方法が知られている(例えば、米国特許第5807522号を参照)。そこに被検体試料から抽出したmRNAを蛍光物質などで標識し、ハイブリダイズさせ、遺伝子がどの様な組織の細胞で高発現しているかを解析することが可能である。またチップ、アレイ上に張り付けるDNAは、本発明の核酸またはその断片をプローブとして用いたPCRの反応産物であってもよい。別法として、本発明の核酸片(DNA断片)を基板上で直接合成してDNAチップもしくはアレイとすることもできる(例えば、米国特許第5424186号を参照)。

#### (4) 遺伝子のクローニング

25 本発明の核酸またはその断片を使用することによってヒト神経芽細胞腫において発現している遺伝子をクローニングすることが可能である。例えば、本発明の

核酸またはその断片をノザンハイブリダイゼーションまたはコロニーハイブリダイゼーションのプロープ、或いはPCRのプライマーとして使用し、本発明の核酸またはその断片を含む遺伝子をクローニングすることが可能である。このようなクローニングの対象となる遺伝子としては、特に予後良好な神経芽細胞腫と予後不良な神経芽細胞腫との間で発現量に差がある遺伝子、他の組織や癌細胞での発現様式とは異なって発現している遺伝子、細胞周期依存的に発現している遺伝子、神経分化に伴って誘導される遺伝子、癌遺伝子または癌抑制遺伝子によって発現が制御される遺伝子等が挙げられる。クローニングは、通常の遺伝子組換え技術に従い、本発明の核酸(DNA)またはその断片を適当なプラスミド、バクテリオファージに組み込み、発現ベクターを構築し、これを宿主細胞に導入して形質転換(導入)し、形質転換体を培養することによって行われる。かかる個々の操作は、例えば、サンブルックら、Molecular Cloning:A Laboratory Manual (前掲)その他、周知の文献に詳述されている。

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(5)腫瘍の予後同定の方法およびそのために使用可能な腫瘍マーカー

上述のように本発明の核酸に関連する遺伝子は、予後良好なヒト神経芽細胞腫で発現が増強されていた。そこで、本発明の核酸(DNA)またはその断片をハイブリダイゼーションのプローブ或いはPCRのプライマーとして使用し、被験者から採取した、臨床組織を含む試料中で、前記遺伝子の発現の増強の有無を調べることにより予後の同定が行える。遺伝子の検出方法としては、前述のノーザンブロットハイブリダイゼーション法、インサイチュハイブリダイゼーション法、およびRT-PCR法等が挙げられる。

ハイブリダイゼーション法を用いるとき、試料中で前記プローブとハイブリダイズする核酸の量が増強する場合、予後が良好であると診断できる。また、RT - PCR法を用いるとき、試料からmRNAを抽出し、これをDNAに逆転写して、前記プライマーにより増幅し、遺伝子発現を半定量的に測定する。このよう

にして遺伝子発現の増強が認められる場合、予後が良好であると診断できる。この特定の診断目的のためには、該プライマーを必須成分として一組含有する診断用キットを用いることが好ましい。該診断用キットは、プライマー成分以外に、PCR用の緩衝液、洗浄液、および酵素等の公知の成分を含む。

5 (6) アンチセンスオリゴヌクレオチド

本発明の別の実施の形態に従えば、本発明の核酸に対するアンチセンスオリゴヌクレオチドが提供される。前記アンチセンスオリゴヌクレオチドは、本発明の核酸にハイブリダイズすることが可能であり、アンチセンスDNAとアンチセンスRNAとを含む。アンチセンスDNAは、DNAからmRNAへの転写を阻害し、アンチセンスRNAは、mRNAの翻訳を阻害する。このようなアンチセンスオリゴヌクレオチドは、天然型であれば自動合成機を使用して、または本発明の核酸を鋳型とするPCR法により合成できる。さらに、該アンチセンスオリゴヌクレオチドは、目的DNAやmRNAとの結合力、組織選択性、細胞透過性、ヌクレアーゼ耐性、細胞内安定性が高められたアンチセンスオリゴヌクレオチド誘導体をも包含する。このような誘導体は、公知のアンチセンス技術を用いて、合成することができる。

mRNAの翻訳開始コドン付近、リボソーム結合部位、キャッピング部位、スプライス部位の配列に相補的な配列を有するアンチセンスオリゴヌクレオチドは、該RNAの合成を阻止することができ、特に遺伝子の発現抑制効果が高い。従って、本発明は、かかるアンチセンスオリゴヌクレオチドを好適に包含する。

#### (7) 遺伝子治療

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本発明の別の実施の形態に従えば、遺伝子治療に用いられる治療用遺伝子をコードする核酸配列が提供される。そこで、本発明の核酸を遺伝子運搬に使用されるベクターに導入して、任意の発現プロモーターにより導入遺伝子(本発明に係る遺伝子)を発現させ、例えば癌の遺伝子治療に用いることができる。

1. ベクター

導入されうるウイルスベクターは、DNAまたはRNAウイルスをもとに作製 できる。MoMLVベクター、ヘルペスウイルスベクター、アデノウイルスベク ター、AAVベクター、HIVベクター、SIVベクター、センダイウイルスベ クター等のいかなるウイルスベクターであってもよい。また、ウイルスベクター の構成タンパク質群のうち1つ以上を、異種ウイルスの構成タンパク質に置換す る、もしくは、遺伝子情報を構成する核酸配列のうち一部を異種ウイルスの核酸 配列に置換する、シュードタイプ型のウイルスベクターも本発明に使用できる。 例えば、HIVの外皮タンパク質であるEnvタンパク質を、小水痘性口内炎ウ イルス(Vesicular stomatitis Virus:VSV)の 外皮タンパク質であるVSV-Gタンパク質に置換したシュードタイプウイルス ベクターが挙げられる [Naldini L等:Science 272 3-(1996)]。さらに、治療効果を持つウイルスであれば、ヒト以外の宿主 域を持つウイルスもウイルスベクターとして使用可能である。ウイルス以外のベ クターとしてはリン酸カルシウムと核酸の複合体、リポソーム、カチオン脂質複 合体、センダイウイルスリポソーム、ポリカチオンを主鎖とする高分子キャリア 一等が使用可能である。さらに遺伝子導入系としてはエレクトロポレーション、 遺伝子銃等も使用可能である。

### 2. 発現プロモーター

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さらに、治療用遺伝子に用いられる発現カセットは、標的細胞内で遺伝子を発現させることができるものであれば、特に制限されることなくいかなるものでも用いることができる。当業者はそのような発現カセットを容易に選択することができる。好ましくは、動物由来の細胞内で遺伝子発現が可能な発現カセットであり、より好ましくは、哺乳類由来の細胞内で遺伝子発現が可能な発現カセットであり、特に好ましくは、ヒト由来の細胞内で遺伝子発現が可能な発現カセットである。発現カセットに用いられる遺伝子プロモーターは、例えばアデノウイルス、サイトメガロウイルス、ヒト免疫不全ウイルス、シミアンウイルス40、ラウス

肉腫ウイルス、単純ヘルペスウイルス、マウス白血病ウイルス、シンビスウイルス、A型肝炎ウイルス、B型肝炎ウイルス、C型肝炎ウイルス、パピローマウイルス、ヒトT細胞白血病ウイルス、インフルエンザウイルス、日本脳炎ウイルス、JCウイルス、パルボウイルス B19、ポリオウイルス等のウイルス由来のプロモーター、アルブミン、SR $\alpha$ 、熱ショック蛋白、エロンゲーション因子等の哺乳類由来のプロモーター、CAGプロモーター等のキメラ型プロモーター、テトラサイクリン、ステロイド等によって発現が誘導されるプロモーターを含む。

以下、本発明により見いだされた予後良好なヒト神経芽細胞腫において発現が 増強する遺伝子群について、実施例に即してさらに詳しく説明するが、本発明の 技術的範囲はこれらの例に限定されるものではない。

(実施例)

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(製造例1) ヒト神経芽細胞腫からの c D N A ライブラリーの構築

1. <u>試料入手</u>

ヒト神経芽細胞腫の臨床組織試料を手術摘出直後に準無菌的に凍結し、その後 15 - 80℃に保存した。

2. 予後良好な試料の選別

1で得られた試料について予後の検定を以下の指標をもとに行った。

#### 予後良好:

予後不良:

病期1または2

・病期 4

・発症年齢が1歳未満

・発症年齢が1歳以上

・手術後5年以上再発なく生存

・手術後3年以内に死亡

・N-mycの増幅なし

·N-myc増幅あり

上記2つの試料において、N-myc増幅は下記のようにして確認した。

上記1で得られた試料を剃刀で細かく切断し、5m1のTENバッファー (5 0mM Tris-HCL(pH=8.0)/1mM EDTA/100mM N a C1)を加えよくホモジナイズした。この混合液に $750\mu1$ のSDS(10%)、

 $125\mu 1$ のproteinase K(20mg/m1)を加え、軽く混和し、50%で8時間放置した。その後、フェノール・クロロホルム処理を行い、最後にエタノール沈殿により、ゲノムDNAを精製した。 $5\mu g$ の得られたゲノムDNAを制限酵素EcoRI(NEB社製)で完全に消化し、N-mycのプロープを用いてサザンハイブリダイゼーションによりN-myc増幅を調べた。

# 3. 予後良好なヒト神経芽細胞腫の臨床組織からmRNAの調製

上記2において予後良好であると判定されたヒト神経芽細胞腫の臨床組織2~3gをTotal RNA Extraction Kit (QIGEN社製) 用いて処理し、トータルRNAを抽出した。抽出したトータルRNAを、オリゴ d Tセルロースカラム (Collaborative社製)を用いて、poly A構造を有するmRNAのプールを精製した。

# 4. mRNAの脱リン酸化

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上記3において調製した $100\sim200\mu$ gのmRNAのプールを $67.3\mu$ 1の0.1%ジェチルピロカーボネート(DEPC)を含む蒸留滅菌水に溶解させ、 $20\mu$ 1の5 x B A P バッファー [Tris-HC1(500 mM、pH=7.0)/メルカプトエタノール(50 mM)]、 $2.7\mu$ 1のRNasin(40 unit/ $\mu$ 1:Promega社製)、 $10\mu$ 1のBAP(0.25 unit  $\mu$ 1、バクテリア由来アルカリフォスファターゼ:宝酒造社製)を加えた。この混合液を37%で1時間反応させ、mRNAの57、末端の脱リン酸化処理を行った。その後、フェノール・クロロホルム処理を2回行い、最後にエタノール沈殿により、脱リン酸化mRNAのプールを精製した。

# 5. 脱リン酸化mRNAの脱キャップ処理

上記4において調製した脱リン酸化mRNAのプールの全量を $75.3\mu1$ の0.1%DEPCを含む蒸留滅菌水に溶解させ、 $20\mu1$ の5 x TAPバッファー [酢酸ナトリウム(250 mM、pH=5.5)/メルカプトエタノール(50 mM)、EDTA(5 mM、pH=8.0))、 $2.7\mu1$  のRNasin(40

unit/ $\mu$ 1)、 $2\mu$ 1のTAP (Tobacco acid pyropho sphatase: 20unit/ $\mu$ 1)] を加えた。この混合液を37 でで1時間反応させ、脱リン酸化mRNAの5 、末端の脱キャップ処理を行った。この際、キャップ構造を持たない不完全長の脱リン酸化mRNAは脱キャップ処理されず5 、末端は脱リン酸化された状態に留まる。その後、フェノール・クロロホルム処理、エタノール沈殿により、脱キャップmRNAのプールを精製した。

# 6. オリゴ<u>キャップmRNAの</u>調製

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上記5において調製した脱キャップmRNAのプールの全量を $11\mu100$ . 1%DEPCを含む蒸留滅菌水に溶解させ、 $4\mu105$ 'ーオリゴRNA(5'ーAGCAUCGAGUCGGCCUUGGCCUACUGGー3':100ng  $/\mu1$ )、 $10\mu10010$  x 1 i g a t i o n バッファー [T r i s - H C 1 (5 00 mM、p H = 7 . 0 ) / メルカプトエタノール(100 mM)]、 $10\mu10$  塩化マグネシウム(50 mM)、2 .  $5\mu10$  ATP(24 mM)、2 .  $5\mu10$  RNasin(40 unit  $/\mu1$ )、 $10\mu10$  T4 RNA 1 i g a s e (25 unit 10 multical 10 mul

# 7. オリゴキャップmRNAからのDNA除去

上記6において調製したオリゴキャップmRNAのプールを $70.3\mu100.1\%$ DEPCを含む蒸留滅菌水に溶解させ、 $4\mu10$ Tris-HC1 (1M、pH=7.0)、 $5.0\mu10$ DTT (0.1M)、 $16\mu10$ 塩化マグネシウム (50mM)、 $2.7\mu10$ RNasin (40unit $/\mu1$ )、 $2\mu10$ DN ase I (5unit $/\mu1$ : 宝酒造社製)を加えた。この混合液を37%で1

# 8. 1st strand cDNAの調製

5 上記7において調製したDNA (-) オリゴキャップmRNAのプールをSu per Script II (ライフテックオリエンタル社製キット) を用いて 逆転写し、1st strand cDNAのプールを得た。DNA (-) オリ ゴキャップmRNAのプールを $21\mu1$ の滅菌蒸留水に溶解させ、 $10\mu1$ の1 OxFirst Strandバッファー (キット付属品)、8μ1のdNTP 10 mix(5mM)、キット付属品)、 $6\mu1$ のDTT(0.1M、キット付属品)、 2.  $5\mu$ 1のオリゴーd Tアダプタープライマー ( $5pmo1/\mu1$ 、5'-GTTTTT-3'), 2.  $0\mu 1\sigma R Nasin (40unit/\mu 1), 2\mu 1\sigma$ Super Script II RTase (キット付属品) を加えた。この 15 混合液を42℃で3時間反応させ、逆転写反応を行った。その後、フェノール・ クロロホルム処理、アルカリ処理、中和処理にて全てのRNAを分解し、エタノ ール沈殿で精製した。

# 9.2nd strand cDNAの調製

上記8において調製した1st strand cDNAのプールをGene
20 Amp (パーキンエルマー社製キット)を用いて、PCR増幅を行った。1s
t strand cDNAのプールを52.4μ1の滅菌蒸留水に溶解させ、
30μ1の3.3xReactionバッファー(キット付属品)、8μ1のdN
TP mix (2.5mM、キット付属品)、4.4μ1の酢酸マグネシウム (2
5mM、キット付属品)、1.6μ1のプライマーF (10pmo1/μ1、5'
-AGCATCGAGTCGGCCTTGTTG-3')、1.6μ1のプライマーR (10pmo1/μ1、5'-GCGCTGAAGACGGCCTATGT

-3')、 $2\mu1$ のrTth (キット付属品)を加えた。この混合液に、 $100\mu1$ のミネラルオイルを静かに加え重層した。この反応液を94%で5分間変性させた後、94%、1分間・<math>52%、1分間・<math>72%、10分間を1サイクルとして<math>12サイクル繰り返し、さらに72%で10分間放置しPCR反応を行った。その後、フェノール・クロロホルム処理、エタノール沈殿で精製し、<math>2nds

5 その後、フェノール・クロロホルム処理、エタノール沈殿で精製し、2 nd s t rand c DNAのプールを得た。

# 10. 2nd strand cDNAのSfiI処理

上記9において調製した2nd strand cDNAのプールを87μ1 の滅菌蒸留水に溶解させ、10×NEBバッファー(NEB社製)、100xBS 10 A(ウシ血清アルブミン、NEB社製)、2μ1のSfiI(制限酵素、20unit/μ1、NEB社製)を加えた。この混合液を50℃で一晩反応させ、SfiIによる制限酵素処理を行った。その後、フェノール・クロロホルム処理、エタノール沈殿で精製し、両末端がSfiI処理されたcDNAのプールを得た。

# 11. SfiI処理されたcDNAのサイズ分画

上記10において調製したSfiI処理されたcDNAのプールを1%のアガロースゲルで電気泳動し、2kb以上の分画をGene clean II(Bio 101社製)を用いて精製した。精製したcDNAのプールは100 $\mu$ 1 の滅菌蒸留水に溶解させ、37℃で6時間放置した。その後、フェノール・クロロホルム処理、エタノール沈殿で精製し、長鎖cDNAのプールを得た。

# 20 12. <u>c D N A ライブラリー</u>

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上記11において調製した長鎖 c D N A のプールを D N A Ligation kit ver. 1 (宝酒造社製キット)を用いてクローニングベクターである p M E 18 S - F L 3 (東京大学医科学研究所 菅野純夫教授より供与)にライゲーションを行った。長鎖 c D N A のプールを  $8\mu$  1 の滅菌蒸留水に溶解させ、あらかじめ制限酵素 D r a I I I で処理された  $1\mu$  1 の p M E 18 S - F L 3、8  $0\mu$  1 の S o lution A (キット付属品)、 $10\mu$  1 の S o lution

B (キット付属品)を加え、16℃で3時間反応させた。その後、フェノール・ クロロホルム処理、エタノール沈殿で精製しcDNAライブラリーを得た。 (実施例2)大腸菌へのトランスフォーメーション

# 1. クローニング

実施例1の12で調製したcDNAライブラリーを大腸菌(TOP-10、Invitrogen社製)にトランスフォーメーションした。cDNAライブラリーを10μ1の滅菌蒸留水に溶解し、TOP-10に混合した。その後、氷上にて30分間、40℃で1分間、氷上で5分間インキュベートした。500μ1のSOB培地を加え、37℃で60分間振盪培養した。アンピシリンを含む寒天培地上に適量づつ播種し、37℃で一昼夜培養して、大腸菌クローンを得た。

# 2. 大腸菌クローンの保存(グリセロールストックの調製)

上記1において得られた寒天培地上の大腸菌クローンを、爪楊枝にて拾い上げ、 96 穴プレートに準備した  $120\mu1$  の L B 培地中に懸濁させた。この 96 穴プレートを 37 でで一晩静置し大腸菌の培養を行った。その後 60% グリセロール 溶液を  $72\mu1$  加え、 -20 で保存した(グリセロールストック)。

(実施例3)核酸配列決定

#### 1. プラスミドの調製

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実施例2の2で調製した10μlのグリセロールストックを15mlの遠心チューブに移し、3mlのLB培地、50μg/mlのアンピシリンを加え、37℃で一晩振盪し、大腸菌の培養を行った。その後、QIAprep Spin Miniprep Kit (QIAGEN社製)を用いて大腸菌からプラスミドDNAを抽出、精製した。

# 2. 両末端シークエンスの解析

上記1において調製したプラスミドDNAをDNA Sequencing Kit (ABI社製キット)を用いて両末端のシークエンスを決定した。 600 ngのプラスミドDNA、8 $\mu$ 1のプレミックス (キット付属品)、3.2 pmo

1のプライマーを混合し、滅菌蒸留水で合計  $20\mu1$ になるように調製した。この混合液を 96%で 2分間変性させた後、 96%、 10 秒間・50%、 5 秒間・60%、 4 分間を 1 サイクルとして 25 サイクル繰り返し反応を行った。その後エタノール沈殿で精製した。変性条件下でポリアクリルアミドゲルにて電気泳動を行い、 ABI377 (ABI社製)を用いて配列決定を行った。

(実施例4) データベースを用いたホモロジー検索

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実施例3において両末端シークエンスを解析して得られた試料の核酸配列情報についてインターネットを介したDNA配列のホモロジー検索を行った。検索にはNCBI(National Center of Biotechnology Information USA, http://www.ncbi.nlm.nih.gov/BLAST)のBLASTを用いた。

(実施例5)半定量的PCRによる予後良好・不良ヒト神経芽細胞腫での遺伝子 発現量の比較

実施例 4 において得られた、遺伝子群の一部から得られた核酸の配列に基づき、 P C R プライマーを合成し、ヒト神経芽細胞腫の予後良好・不良の臨床組織で発現量を比較定量した。実施例  $1 \sim 3$  に示した方法(R T - P C R)で前記ヒト神経芽細胞腫の臨床組織から m R N A を抽出し、 r T a q(宝酒造社製)を用いて P C R 反応を行った。具体的には、  $5 \mu 1$  の滅菌蒸留水、  $2 \mu 1$  のm R N A、  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 0 \times r$  T a q  $1 \mu 1$  の  $1 \times r$  で  $1 \times r$  で  $1 \times r$  を  $1 \times r$  で  $1 \times r$  の  $1 \times r$  を  $1 \times r$  の  $1 \times r$  の

うち73個がホモロジー無しであった。)を含め、配列番号 $1\sim104$ に記載する 核酸配列および核酸の情報を表 $1\sim2$ に示す。

また、半定量的PCRによる予後良好・不良ヒト神経芽細胞腫での遺伝子発現量の測定結果の一例(核酸配列nbla-00106、nbla-00219、nbla-03145について)を図 $1\sim3$ に示す。

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【表1】 予後良好な神経芽細胞腫で発現が増強している核酸配列

配列番号		一細胞周期特異性	ホモロジー(Accession No.)
1	nbla-00002		KIAA0327(AB002325)
2	nbla-00012	S期	
3	nbla=00052		_
	nbla-00067		
	nbla-00078	S期	KIAA0322(AB002320)
_	nbla-00086-f	10,01	GTPaseRAB6B(AF166492)
	nbla=00086-r	+	GTF aser(AD0D(AF 100492)
	nbla=00100	G2/M期	VIA 40620/4 DO1 4520)
	nbla-00106	GZ/ WIAH	KIAA0632(AB014532)
	nbla-00108	<del></del>	—
		<del></del>	KIAA0874(AB020681)
	nbla-00118	<del></del>	-
	nbla-00126		MAB21L1(NM_005584)
-	nbla-00137		_
	nbla-00150	G2/M期	SART-3(AB020880)
	nbla=00158		<u> </u>
	nbla-00172	G2/M期	
17	nbla=00177	S期	_
18	nbla-00204		
19	nbla-00219		KIAA0367(AB002365)
20	nbla-00235	G2/M期	
	nbla-00237		
	nbla-00271	<u> </u>	KIAA0886(AB020693)
	nbla-00343		KIAA1145(AB032971)
	nbla-00371	S期	
	nbla-00375	[O79]	
	nbla=00375	<del> </del>	<del></del>
	nbla=00433		
	nbla=00433	S期及びG2/M期	
			T1 00711/DE0505)
	nbla-00490	G2/M期	T1-227H(D50525)
	nbla=00538-f	<del> </del>	DKFZp566D1146(AL080222)
	nbla-00538-r		DKFZp566D1146(AL080222)
	nbla-00613		
	nbla-00650		
	nbla-00652		FLJ10739 fis(AK001601)
	nbla-00660	G2/M期	
	nbla-00693		DKFZp434G0827(AL122107)
37	nbla-00697	G1期及びS期	
38	nbla-00715		
39	nbla=00744		_
40	nbla-00761	S期	KIAA0751(AB018294)
41	nbla=00830-f		
42	nbla-00830-r		_
	nbla=00831=f		KIAA0868(AB020675)
	nbla=00831=r		KIAA0868(AB020675)
	nbla=00832=f	<del>                                     </del>	
	nbla-00832-r	† <del></del>	(AF140710)
	nbla-02942		(NM 001788)
	nbla-02942 nbla-02975	G1期	
		UI #I	FLJ10103 fis(AK000965)
	nbla-02981	00 /0 4 HD	<u> </u>
	nbla=02999	G2/M期	(AF182814)
	nbla-03010	G1期	
	nbla-03103	G1期	
	nbla-03107-f		KIAA1309(AB037730)
	nbla=03107-r	,	KIAA1309(AB037730)

差 替 え 用 紙 (規則26)

# 【表2】

	nbla-03139	S期及びM期	FOG2(NM_012082)
	nbla=03145	G1期	XCE(Y16187)
57	nbla-03199-f	S期	<b> -</b>
58	nbla-03199-r	S期	
59	nbla-03212-f	S期	_
60	nbla-03212-r	S期	_
	nbla=03219-f		
	nbla=03219=r		
	nbla=03301-f	S期	NF-L(X05608)
	nbla=03301-r	S期	
	nbla=03461-f	0,41	
	nbla=03461-r		
	nbla=03539-f	S期	
		S期	
	nbla-03539-r		— (4.4.4.054.3/4.5.044.000)
	nbla=03575-f		KIAA0517(AB011089)
	nbla-03575-r	S期及びG2/M期	
	nbla-03646-f		KIAA0018(D13643)
	nbla-03646-r		KIAA0018(D13643)
	nbla-03684-f		
	nbla-03755-r	S期	
	nbla-03759-f	_	
76	nbla=03759-r		
77	nbla-03761-f		
78	nbla=03761=r		
79	nbla-03771-f		
80	nbla=03771-r	-	_
81	nbla-03777-f		_
82	nbla=03777-r	·	_
83	nbla-03779-f		
84	nbla=03779-r		_
	nbla-03781-f		_
	nbla-03781-r	-	DKFZp434C035(AL137633)
	nbla-03831-f		_
	nbla=03831=r		
	nbla-03851-f		
	nbla=03851=r		
	nbla-03862-f		
	nbla=03862=r		
	nbla=03898-f		
	nbla-03898-r		
	nbla-03911-f		
	nbla-03911-r		<del>_</del>
	nbla-03914-f		
	nbla-03914-r		
	nbla-04021-f		
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差 替 え 用 紙 (規則26)

(実施例6) 半定量的PCRによる細胞周期依存的遺伝子発現量の測定

実施例4において得られた、遺伝子群の一部から得られた核酸の配列に基づき、 PCRプライマーを合成し、HeLa細胞を用いて、細胞周期特異的な遺伝子発 現量を比較定量した。HeLa細胞はそれぞれ以下のように処理を行った。

5 (1)無処理

15

20

- (2)  $400\mu$  Mのmimosineで18時間処理し、65%の細胞がG1期の状態
- (3) 2 mMのthymidineで20時間処理し、100%の細胞がS期の 状態
- 10 (4) 0.6μg/mlのNocodazoleで18時間処理し、85%の細胞がG2/M期の状態

#### 産業上の利用可能性

25 本発明の核酸は、神経芽細胞腫において発現する遺伝子の情報を明らかにする。 本発明の核酸またはその断片は、プローブ或いはプライマーとして、各種ハイ

ブリダイゼーションまたはPCR法に使用でき、前記遺伝子の他組織、細胞での発現の検出や、その構造および機能の解析を可能とする。また、該遺伝子がコードするヒト蛋白の遺伝子工学的製造も可能となる。

また、本発明の核酸は、予後良好なヒト神経芽細胞腫と、予後不良なヒト神経芽細胞腫とを比較したとき、予後良好なヒト神経芽細胞腫で発現が増強されている遺伝子に由来する核酸であり、従って、これらの核酸に基づく遺伝子情報により神経芽細胞腫の予後の診断が可能となる。該遺伝子は、N-myc遺伝子が予後不良因子であるのに対して、TrkA遺伝子と同様に予後良好因子と見なされるので、神経芽細胞腫の悪性度および抗癌剤に対しての感受性の指標(腫瘍マーカー)となり得る。

5

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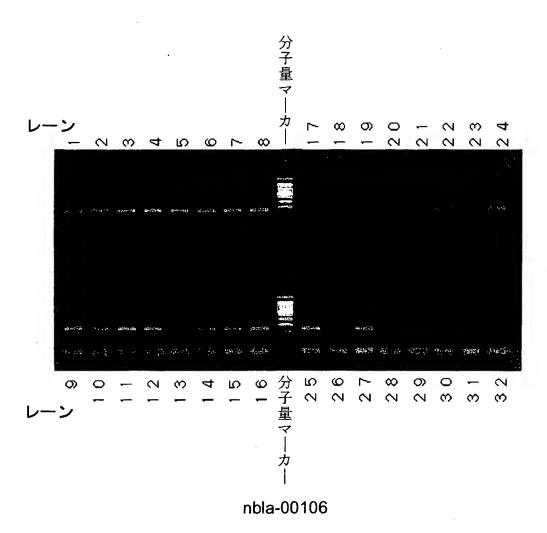
# 請求の範囲

1. ヒト神経芽細胞腫において発現する遺伝子に由来する核酸であって、配列表の配列番号1ないし104に記載の核酸配列からなる群より選ばれる1つの配列からなることを特徴とする核酸、またはそれに相補的な核酸。

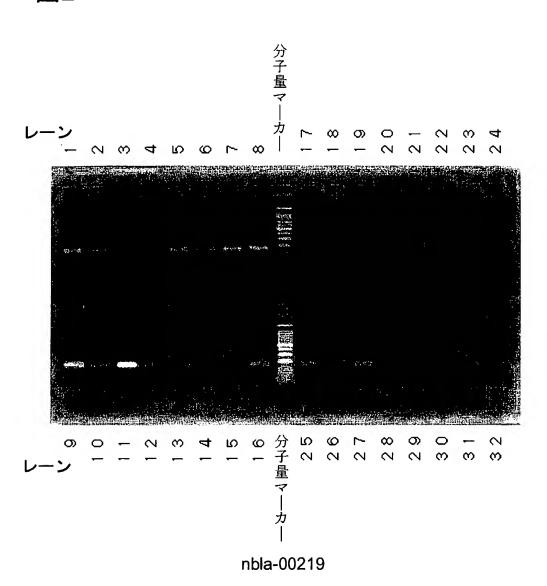
- 5 2. 前記核酸がDNAであることを特徴とする請求項1に記載の核酸。
  - 3. 予後良好なヒト神経芽細胞腫と、予後不良なヒト神経芽細胞腫との比較において、予後良好なヒト神経芽細胞腫で発現が増強されている遺伝子に由来し、配列表の配列番号1ないし104に記載の核酸配列からなる群より選ばれる1つの配列からなることを特徴とする核酸、またはそれに相補的な核酸。
- 10 4. 前記核酸がDNAであることを特徴とする請求項3に記載の核酸。
  - 5. 請求項1~4のいずれか1つの項に記載の核酸の断片。

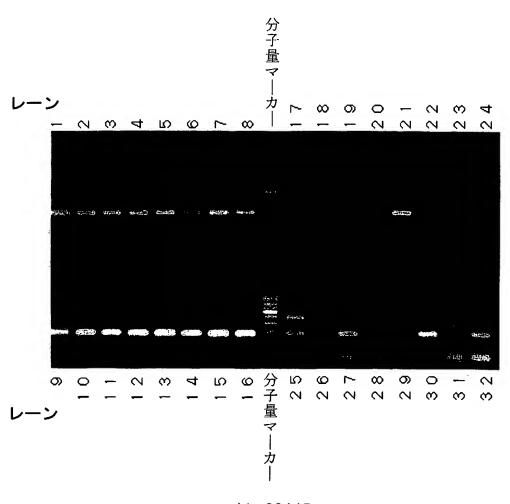
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- 6. 請求項1~4のいずれか1つの項に記載の核酸とストリンジェントな条件下でハイブリダイズすることを特徴とする、単離された核酸。
- 7. 前記核酸がDNAであることを特徴とする請求項6に記載の単離された核酸。
  - 8. 請求項7に記載の核酸からなることを特徴とする P C R プライマー。
  - 9. 請求項3に記載の核酸をヒト神経芽細胞腫の臨床組織から検出することを 特徴とする、ヒト神経芽細胞腫の予後の診断方法。
- 10. 請求項8に記載のPCRプライマーの一組を含むことを特徴とするヒト **20** 神経芽細胞腫の予後の診断用キット。

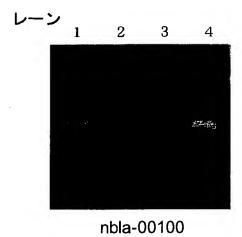


1/4





nbla-03145



SEQUENCE LISTING

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1

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13

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<211> 2254

<212> DNA

<213 > Homo sapiens

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<213> Homo sapiens

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<213 > Homo sapiens

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<211> 2214

<212> DNA

<213> Homo sapiens

<400> 27

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<211> 2016

<212> DNA

<213> Homo sapiens

<400> 28

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<212> DNA

<213 > Homo sapiens

<400> 29

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<sup>&</sup>lt;210> 30

<sup>&</sup>lt;211> 865

<sup>&</sup>lt;212> DNA

<sup>&</sup>lt;213> Homo sapiens

<400> 30

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<211> 876

<212> DNA

<213> Homo sapiens

<400> 31

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<210> 32

<211> 2274

<212> DNA

<213> Homo sapiens

<400> 32

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<210> 33

<211> 2465

<212> DNA

<213 > Homo sapiens

<400> 33

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<211> 2280

<212> DNA

<213 > Homo sapiens

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<211> 2404

<212> DNA

<213> Homo sapiens

<400> 35

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<211> 1690

<212> DNA

<213 > Homo sapiens

<400> 36

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<211> 2963

<212> DNA

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<213> Homo sapiens

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<211> 772

<212> DNA

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<211> 782

<212> DNA

<213> Homo sapiens

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<211> 762

<212> DNA

<213> Homo sapiens

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<211> 793
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<211> 774

<212> DNA

<213> Homo sapiens

<400> 46

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<210> 47

<211> 2415

<212> DNA

<213 > Homo sapiens

<400> 47

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<211> 2362

<212> DNA

<213 > Homo sapiens

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<212> DNA

# <213 > Homo sapiens

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<211> 3457

<212> DNA

<213 > Homo sapiens

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<211> 2158

<212> DNA

<213> Homo sapiens

<400> 51

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<210> 52

<211> 2142

<212> DNA

<213> Homo sapiens

<400> 52

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<210> 53

<211> 846

<212> DNA

<213 > Homo sapiens

<400> 53

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<210> 54

<211> 836

<212> DNA

<213 > Homo sapiens

<400> 54

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<210> 55

<211> 3415

<212> DNA

<213 > Homo sapiens

<400> 55

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<210> 56

<211> 1829

<212> DNA

<213 > Homo sapiens

# <400> 56

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<210> 57

<211> 778

<212> DNA

<213> Homo sapiens

<400> 57

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ncctannann	accnnacnac	cnnaccccac	anacannaac	aacccacaaa	tannccnaca	540
nnannentea	cnacaannnc	aacgnantcn	caaaanaccc	ccncaannnn	nanaannaca	600
ccacaacana	nnaaaacnan	aacnantaac	anaaaaanac	naaaaanaan	accccaatcn	660
caccacaaaa	cacnncacaa	nncccccana	atnncaccct	caccncacaa	acaaacnacc	720
accacaaaac	aaanannaan a	aaaaaaanca a	aaccanenn aa	atnacaaac aa	aacncg 778	

<210> 58

<211> 753

<212> DNA

<213 > Homo sapiens

# <400> 58

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naatgcacta teetacagca gtetggttgg tnaatteang nacttintga geeangggaa 660 aaaaaagtaa eetggttggt tgaaggettg ganaateaag ggtganaent ntnattengn 720 tnggengett tgggeeecat taaaaaggee ggg 753

<210> 59

<211> 766

<212> DNA

<213> Homo sapiens

<400> 59

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<210> 60

<211> 750

<212> DNA

# <213> Homo sapiens

## <400> 60

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<210> 61

<211> 756

<212> DNA

<213 > Homo sapiens

# <400> 61

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tegggacage gagegeagee aggttetete teeteggae agggtggte eegtteegtg 360 catteeceag etgeageea gagaaacaat ttggagega acceggete tgaeeteece 420 teateeteag eetteecea gggatggee gtgagatgaa tgtggteace ggceeaatee 480 aagggtetat ggecaaaceg eagaeeegga ggaageagge eaggeeatet ggggageegg 540 etteeettet etteteetg eteeacaaag etgetetate eagaageeag gecegeetgt 600 gageaagggg aggetgeang tgtteettea eetgaagegt gtgaaageea acaggeeeca 660 eeetggtete ageegnagee eetteeagae teanggggee aaaceaettt teacageeat 720 tgtaaceaaa egtntggeea eaetttgnte gaetea 756

<210> 62

<211> 799

<212> DNA

<213 > Homo sapiens

<400> 62

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## acatacanac ngaaacccg 799

<210> 63

<211> 796

<212> DNA

<213 > Homo sapiens

# <400> 63

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<210> 64

<211> 821

<212> DNA

<213 > Homo sapiens

<400> 64

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<210> 65

<211> 738

<212> DNA

<213 > Homo sapiens

<400> 65

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ctgggagaga gaggcgttct cacaatgcct tgaaaatggg aactttgcat cctttaaatt 360 tttccaaact gacttagttt gtttaccttg aatttctggg atggggcaaa tgtgaccttc 420 atgctatagg gcccacgttt ccagatttgg tatggaaaga aggaagaaag tctgaccctc 480 ttgnttttaa gataggcaaa aggaagatga gatagtccat ggttcaccac ccaangnect 540 tctgggcact ggctgggctg acgctgggcc tggttccagc tatgcctacc tttctttgc 600 cataccacac cgttgcttta tgagcattct tttggtaagg ncaagatcaa gataaccttt 660 ttccttgaa taataggaca agcacctttc ccagtgggcc tttaatggca tctgaatgtn 720 naaagggaaa ccaccctt 738

<210> 66

<211> 745

<212> DNA

<213> Homo sapiens

#### <400> 66

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<210> 67
<211> 739
<212> DNA
<213> Homo sapiens
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<400> 67

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<210> 68

<211> 747

<212> DNA

<213> Homo sapiens

<400> 68

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actggcccca	gggactcagc	ctgctttcct	atccatcccc	tcagtagctg	tcaccatgca	180
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gaagcccaaa	cctttgggtt	ttaaggagtt	tattgctaat.	ctgtaaaaca	gaaagagaca	300
ggagataagc	atgacaaaat	atagggaaga	aatgactttt	gcctaaactt	ccaattgtgt	360
acaattgaag	cctctgcttt	atagctctta	gcacacctct	caaataagaa	ggcaagtact	420
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naaaaacttn	aaaacactac	tgganaaaaa	aggtctcngg	aaggngatga	aancentnac	720
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<210> 69

<211> 726

<212> DNA

<213> Homo sapiens

# <400> 69

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<210> 70

<211> 854

<212> DNA

<213> Homo sapiens

<400> 70

<210> 71

<211> 728

<212> DNA

<213 > Homo sapiens

<400> 71

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tgggtgcgcc	tgaaggggct	ggagttcgct	tatcttcgat	atctactact	acgtgcgcgc	180		
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caagaaan 728								

<210> 72

<211> 740

<212> DNA

<213> Homo sapiens

<400> 72

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<210> 73

<211> 761

<212> DNA

<213 > Homo sapiens

## <400> 73

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<210> 74

<211> 783

<212> DNA

<213> Homo sapiens

<400> 74

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<210> 75

<211> 761

<212> DNA

<213 > Homo sapiens

<400> 75

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atatttccat gtaaatctag atctttggag caattaagat ggaattacaa tttctaggga 180
gcattttaag gaaaatgttt tggcttttc ataattttat gtcttacagt atggaattat 240
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<210> 76

<211> 788

<212> DNA

<213> Homo sapiens

<400> 76

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gaataagn 788							

<210> 77

<211> 738

<212> DNA

<213> Homo sapiens

## <400> 77

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<210> 78

<211> 785

<212> DNA

<213> Homo sapiens

<400> 78

<210> 79

<211> 774

<212> DNA

<213> Homo sapiens

<400> 79

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<210> 80

<211> 784

<212> DNA

<213> Homo sapiens

<400> 80

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<210> 81

<211> 782

<212> DNA

<213> Homo sapiens

# <400> 81

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<210> 82

<211> 788

<212> DNA

<213> Homo sapiens

<400> 82

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<210> 83

<211> 780

<212> DNA

# <213> Homo sapiens

## <400> 83

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tteeetteet eteeettea ettnaeecae aatettgaaa aaettnettt etettetgng 660
aacateattg geeagateea tttteaatgg netggateet tttaattee ttteaaettg 720
aaagaaaetg gaeattagge actatgnggt gggtaetgee etantggtea agtgeetett 780

<210> 84

<211> 792

<212> DNA

<213> Homo sapiens

## <400> 84

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<210> 85

<211> 787

<212> DNA

<213> Homo sapiens

# <400> 85

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<210> 86

<211> 789

<212> DNA

<213> Homo sapiens

<400> 86

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<210> 87

<211> 766

<212> DNA

### <213> Homo sapiens

<400> 87

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<210> 88

<211> 785

<212> DNA

<213> Homo sapiens

<400> 88

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<210> 89

<211> 717

<212> DNA

<213> Homo sapiens

### <400> 89

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<210> 90
<211> 726
<212> DNA
<213> Homo sapiens
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<400> 90

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<210> 91

<211> 722

<212> DNA

<213> Homo sapiens

<400> 91

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<210> 92

<211> 724

<212> DNA

<213 > Homo sapiens

<400> 92

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<210> 93

<211> 758

<212> DNA

<213> Homo sapiens

<400> 93

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<210> 94

<211> 758

<212> DNA

<213> Homo sapiens

<400> 94

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<210> 95

<211> 747

<212> DNA

<213> Homo sapiens

<400> 95

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<210> 96

<211> 768

<212> DNA

<213> Homo sapiens

<400> 96

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<210> 97

<211> 750

<212> DNA

<213> Homo sapiens

<400> 97

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<211> 740

<212> DNA

<213 > Homo sapiens

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<212> DNA

<213 > Homo sapiens

<400> 102

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<210> 103

<211> 734

<212> DNA

<213 > Homo sapiens

<400> 103

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<210> 104

<211> 738

<212> DNA

<213> Homo sapiens

<400> 104

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/01631

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl <sup>7</sup> C12N15/12, C12Q1/68						
According to International Patent Classification (IPC) or to both national classification and IPC						
	S SEARCHED					
Minimum documentation searched (classification system followed by classification symbols)  Int.Cl <sup>7</sup> C12N15/12, C12Q1/68						
	Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Genbank/EMBL/DDBJ/GeneSeq, WPI (DIALOG), BIOSIS (DIALOG)						
C. DOCU	MENTS CONSIDERED TO BE RELEVANT					
Category*	Citation of document, with indication, where a		Relevant to claim No.			
А	Takahiro N et al., "Prediction of Unidentified Human Genes.VII of 100 New cDNA Clones from Brain Proteins in vitro", DNA Res. (pp.141-150	I.The Complete Sequences Which Can Code for Large	1-10			
A	Takemasa K et al., "Multistep neurogenic tumors", Molecular No.4, pp.366-372		1-10			
	documents are listed in the continuation of Box C.	See patent family annex.				
Special categories of cited documents:  "A" document defining the general state of the art which is not considered to be of particular relevance earlier document but published on or after the international filing date  "E" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  "O" document referring to an oral disclosure, use, exhibition or other means  "P" document published prior to the international filing date but later than the priority date claimed  Date of the actual completion of the international search 16 May, 2001 (16.05.01)  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention cannot be considered novel or cannot be considered novel or cannot be considered novel or cannot be considered to involve an inventive step when the document is document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document of particular relevance; the claimed invention combined with one or more other such document, such combination being obvious to a person skilled in the art document member of the same patent family  Date of mailing of the international search report 29 May, 2001 (29.05.01)			e application but cited to arlying the invention laimed invention cannot be ed to involve an inventive laimed invention cannot be when the document is documents, such skilled in the art amily			
	ailing address of the ISA	Authorized officer				
Japanese Patent Office						
Facsimile No.		Telephone No.				

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/01631

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:    Claims Nos: because they relate to subject matter not required to be searched by this Authority, namely:    Claims Nos: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:    Claims Nos: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).    Box II Observations where unity of Invention is tacking (Continuation of Item 2 of Ifrest sheet)   This International Searching Authority found multiple inventions in this international application, as follows:    In claims 1 to 8 and 10, inventions relating to 104 nucleic acids originating in human neuroblastoma, which are different from each other in base sequence, are described in a single claim.    At the filling date of the present application, the nucleic acid of an oncogene expressed specifically in neuroblastoma was already publicly known and the relation thereof to the prognostic conditions (benign or acritical) of neuroblastoma was also publicly known.    Such being the case, there is no technical relationship among the claimed inventions involving any "special technical feature".    Thus, these claims are considered as not complying with the requirement of unity of invention.    As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.    As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fees were paid, specifically claims Nos:    As all searchable claims could be searched without effort justifying an additional fee, this international search report covers only those claims for which fees were timely paid by the applicant. Consequently, t	Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)		
because they relate to subject matter not required to be searched by this Authority, namely:  2. Claims Nos:     because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  3. Claims Nos:     because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).  Box II Observations where unity of invention is tacking (Continuation of Item 2 of first sheet)  This international Searching Authority found multiple inventions in this international application, as follows:     In claims 1 to 8 and 10, inventions relating to 104 nucleic acids originating in human neuroblastoma, which are different from each other in base sequence, are described in a single claim.  At the filing date of the present application, the nucleic acid of an oncogene expressed specifically in neuroblastoma was already publicly known and the relation thereof to the prognostic conditions (benign or acritical) of neuroblastoma was also publicly known.  Such being the case, there is no technical relationship among the claimed inventions involving any "special technical feature".  Thus, these claims are considered as not complying with the requirement of unity of invention.  1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.  2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.  3. No required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  Parts of claims 1 to 8 and 10 concerning SEQ ID No:1.	This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:		
because they relate to subject manter not required to be searched by this Authority, namely:  2. Claims Nos:     because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  3. Claims Nos:     because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).  Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)  This International Searching Authority found multiple inventions in this international application, as follows:     In claims 1 to 8 and 10, inventions relating to 104 nucleic acids originating in human neuroblastoma, which are different from each other in base sequence, are described in a single claim.  At the filing date of the present application, the nucleic acid of an oncogene expressed specifically in neuroblastoma was already publicly known and the relation thereof to the prognostic conditions (benign or acritical) of neuroblastoma was also publicly known.  Such being the case, there is no technical relationship among the claimed inventions involving any "special technical feature".  Thus, these claims are considered as not complying with the requirement of unity of invention.  1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.  2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.  3. No required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos:  Parts of claims 1 to 8 and 10 concerning SEQ ID No:1.			
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because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  3.	because they relate to subject matter not required to be searched by this Authority, namely:		
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  3.			
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In claims 1 to 8 and 10, inventions relating to 104 nucleic acids originating in human neuroblastoma, which are different from each other in base sequence, are described in a single claim.  At the filing date of the present application, the nucleic acid of an oncogene expressed specifically in neuroblastoma was already publicly known and the relation thereof to the prognostic conditions (benign or acritical) of neuroblastoma was also publicly known.  Such being the case, there is no technical relationship among the claimed inventions involving any "special technical feature".  Thus, these claims are considered as not complying with the requirement of unity of invention.  1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.  2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.  3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  Parts of claims 1 to 8 and 10 concerning SEQ ID NO:1.  Remark on Protest   The additional search fees were accompanied by the applicant's protest.			
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A. 発明の属する分野の分類(国際特許分類(IPC)) Int Cl' Cl2N15/12, Cl2Q1/68						
B. 調査を行	テった分野					
	最小限資料(国際特許分類(1 P C))					
Int. C	Int. Cl' Cl2N15/12, Cl2Q1/68					
最小限资料以外	<b>外の資料で調査を行った分野に含まれるもの</b>					
国際調査で使用した電子データベース(データベースの名称、調査に使用した用語) Genbank/EMBL/DDBJ/GeneSeg,WPI(DIALOG),BIOSIS(DIALOG)						
C. 関連する						
引用文献の	2 C BD 0 34 t Q X m		関連する			
カテゴリー*	引用文献名 及び一部の箇所が関連すると	ときは、その関連する箇所の表示	請求の範囲の番号			
A	Takahiro N et al., "Prediction of		1 - 1 0			
A			1-10			
	Unidentified Human Genes. VII. The	-				
	New cDNA Clones from Brain Which					
	<i>in vitro</i> ", DNA Res. (1997) Vol. 4, No.	2, p. 141-150				
Α	Takemasa K et al., "Multistep card	inogenesis of neurogenic	1-10			
	tumors", Molecular Medicine (1999) V	=				
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* 引用文献の	ウカテゴリー	の日の後に公表された文献				
	車のある文献ではなく、一般的技術水準を示す	「T」国際出願日又は優先日後に公表さ	された文献であって			
もの						
「E」国際出願日前の出願または特許であるが、国際出願日の理解のために引用するもの			- y , · // // // // // // // // // // // // /			
以後に公表されたもの 「X」特に関連のある文献であって		当該文献のみで発明				
		の新規性又は進歩性がないと考え	-			
日若しくは他の特別な理由を確立するために引用する 「Y」特に関連のある文献であ						
	里由を付す) トス関ラ (4円 R三体)をデアナスでお	上の文献との、当業者にとって自				
	「O」口頭による開示、使用、展示等に言及する文献 よって進歩性がないと考えられるもの					
「P」国際出願日前で、かつ優先権の主張の基礎となる出願 「&」同一パテントファミリー文献						
国際調査を完了	了した日	国際調査報告の発送日	-			
16. 05. 01		2 9.05.0				
国際調査機関の	の名称及びあて先	特許庁審査官(権限のある職員)	4N 9637			
日本国特許庁(ISA/JP)		本間 夏子				
郵便番号100-8915		· ·	У			
東京都千代田区霞が関三丁目4番3号		電話番号 03-3581-1101	内線 3488			

第1欄	請求の範囲の一部の調査ができないときの意見 (第1ページの2の続き)
法第8条 成しなか	等3項 (PCT17条(2)(a)) の規定により、この国際調査報告は次の理由により請求の範囲の一部について作いた。
1. [	請求の範囲は、この国際調査機関が調査をすることを要しない対象に係るものである。 つまり、
2.	請求の範囲 は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、
3. 🗌	請求の範囲 は、従属請求の範囲であってPCT規則6.4(a)の第2文及び第3文の規定に 従って記載されていない。
第Ⅱ欄	発明の単一性が欠如しているときの意見 (第1ページの3の続き)
次に対	さべるようにこの国際出願に二以上の発明があるとこの国際調査機関は認めた。
腫にそし、な	情求の範囲請求項1-8,10には、それぞれ異なる塩基酸配列を有する104個のヒト神経芽細胞 出由来する核酸に関する発明が1つの請求項中に記載されている。 して、本願出願時神経細胞腫に特異的に発現されている癌遺伝子について公知の核酸が存在 神経細胞腫の予後の良不良の関係についても公知である。 って、クレームされた発明の間には「特別な技術的特徴」を含む技術的な関係を見いだすことは ない。
Ĵ	って、発明の単一性を満たしていないと認められる。
1.	出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求 の範囲について作成した。
2.	追加調査手数料を要求するまでもなく、すべての調査可能な請求の範囲について調査することができたので、追加調査手数料の納付を求めなかった。
3.	出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求の範囲のみについて作成した。
4. 🗵	出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載 されている発明に係る次の請求の範囲について作成した。
	請求項1-8,10における配列番号1に関する部分
追加調查	手数料の異議の申立てに関する注意
	] 追加調査手数料の納付と共に出願人から異議申立てがあった。 ] 追加調査手数料の納付と共に出願人から異議申立てがなかった。

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